INFLUENCE OF WASHING TREATMENT ON NATIVE MICROFLORA AND *ESCHERICHIA COLI* POPULATION OF INOCULATED CANTALOUPES

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ABSTRACT

The influence of chlorine or hydrogen peroxide treatment on populations of Escherichia coli *25922 on the external surface of inoculated cantaloupe was investigated. Surface treatment with 70% EtOH, followed by immersion in 10^8 CFU/mL* E. coli *inoculum deposited an average of 4.4 log_{10}CFU/cm^2 cell population on the cantaloupe surface. The efficacy of washing inoculated cantaloupe was dependent on storage interval between inoculation and treatment. Dipping the cantaloupes in solutions containing 1000 mg/L chlorine or 5% peroxide for 5 min, within 24 h of inoculation, caused a 2 log_{10} CFU/cm^2 reduction of the indigenous surface microflora and a 3-4.0 log_{10} CFU/cm^2 reduction in* E. coli. *The efficacy was less when the interval between inoculation and treatment exceeded 24 h. Chlorine appeared to be a better antimicrobial agent than hydrogen peroxide against* E. coli *ATCC 25922 inoculated on cantaloupe surfaces while hydrogen peroxide was better in reducing surface microflora of cantaloupe.*

INTRODUCTION

There are many reports of disease due to consumption of fruits and vegetables that were contaminated at the surface with enteric pathogens (Geldreich and Bordner 1971; Beuchat 1995). The safety of fresh and fresh-cut produce availabe in salad-bar operations and supermarkets is a concern (Hurst and Schuler 1992).

Enterohemorrhagic *E. coli* O157:H7 is recognized as an important foodborne

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pathogen (Doyle 1991; Madden 1992; Padhye and Doyle 1992). Outbreaks of diarrhea and hemolytic uremic syndrome, associated with *E. coli* O157:H7 in unpasterized apple juice (Besser *et al.* 1993; Anon 1996; CDC 1997), have raised concerns about the adequacy of some sanitation practices and the need for regulatory action (Sapers *et al.* 1999). In August, 1993, an outbreak of foodborne illness was linked to eating cantaloupe contaminated with *E. coli* O157:H7 (M. Diermayer, Oregon Health Division, Portland, Portland, OR, personal communication). In August, 2000, an outbreak of foodborne illness and one death was linked to eating watermelon served on salad bar (S. Foldy, Milwaukee Health Division, WI, The Milwaukee Journal Sentinel). Although the pathogen originated from raw beef that was ground directly next to where ready to eat salad items were being prepared (Beers 2000), the pathogen survived and grew on melon tissue. Minimally processed fresh fruits and vegetables provide a good substrate for microbial growth (Marston 1995; Nguyen-The and Carlin 1994). Such substrate may allow proliferation of human pathogenic organisms like enterotoxigenic *Escherichia coli*. Sanitation and the nature of the microflora are of primary importance to the maintenance, quality, shelf stability and safety of fresh produce (Brackett 1992; Beuchat 1995).

Washing with chlorinated water is recommended to remove soil from fresh produce and reduce the microbial load (Madden 1992). Chlorination of wash water has been reported to prevent microbial contamination in produce processing lines (Yildiz 1994). However, chlorination may result in formation of potentially carcinogenic chlorinated organic compounds (Wei *et al.* 1985), and there is much interest in developing safer sanitizers. In this study, the ability of chlorine and hydrogen peroxide to reduce the population of indigenous microflora and *E. coli* ATCC 25922 attached to cantaloupe surface was investigated. This organism was chosen based on unpublished data (Riordan 2000) that indicated it has similar growth characteristics to *E. coli* O157:H7. Therefore, it would be a good surrogate for this study. The influence of storage time and temperature prior to washing on bactericidal activity of chlorine and hydrogen peroxide were also investigated.

MATERIALS AND METHODS

Test Organism

Escherichia coli ATCC 25922, a nonpathogenic strain, was maintained on brain heart infusion (BHI) agar slants at 4C. The cells were cultivated in tryptic soy broth (TSB, Difco, Detroit, MI), incubated at 35C for 18 h. Cells were harvested by centrifugation at 12,000 g for 10 min at 4C. The cell pellets were washed twice in salt-peptone buffer (0.85% NaCl, 0.05% Bacto-peptone (Difco)), and then suspended in the same buffer. The suspended cells were transferred to 3 L of 0.1% peptone water which was used as the inoculum. The final concentration of *E. coli*

in the inoculum dip, determined by plating 0.1 mL on BHIA, incubated at 35C for 24 h, was 10^8 colony forming units (CFU)/mL.

Preparation of Wash Solutions

Clorox, a commercial bleach containing 5.25% sodium hypochlorite (NaOCl, Clorox Co., Oakland, CA), was diluted in sterile water to provide a concentration of 1000 mg/L of available chlorine in the wash solution. Free chlorine in the solution was determined with a chlorine test kit (Hach Co., Ames, IA) that has been approved by the U.S. Environmental Protection Agency. The pH was adjusted to 6.4 ± 0.1 by adding citric acid. A 5% hydrogen peroxide solution was prepared from a 30% stock solution (Reagent grade, Fisher Scientific, Suwanee, GA).

Cantaloupes

To investigate the effect of sanitizing treatments on bacterial attachment to cantaloupe surfaces, cantaloupes, purchased from a local supermarket, were divided into the following categories: (1) unwashed; (2) washed with tap water; and (3) surface-treated with 70% EtOH for 1 min. Water-washed and surface-treated cantaloupes were air dried for 1 h in a biosafety cabinet. Whole cantaloupes were submerged in 10^4 to 10^9 CFU/mL *E. coli* inoculum for up to 10 min in order to investigate the influence of inoculum size and contact time on bacterial attachment to the cantaloupe surface. An inoculum containing 10^8 CFU/mL *E. coli* and a contact time for 5 min were chosen from this study and used throughout the investigation. Cantaloupes were submerged in 3L of bacterial inoculum and agitated by stirring with glove covered hand for 5 min to ensure even inoculation. After inoculation, the cantaloupes were placed on 70 x 50 crystallizing dish (Pyrex®) inside a biosafety cabinet to dry for 1 h and then stored at 20C or 4C for up to 120 h before sanitizer treatments were applied.

At 0, 24, 72 and 120 h storage time, cantaloupes, inoculated or not inoculated, were dipped in chlorine or hydrogen peroxide solution with agitation for 5 min, and then rinsed with sterile tap water. All treated samples were air dried for 1 h at room temperature before sampling.

Sample Preparation for Microbiology

A sterilized stainless steel cork-borer was used to cut through the cantaloupe surface at random locations to produce rind plugs of 22 mm in diameter with a rind surface area (πr^2) of 3.80 cm^2. Flesh adhering to the rind plugs was trimmed off using a sterilized stainless steel knife.

Microbiological Analysis

Cantaloupe rind plugs (70) weighing approximately 25 g (with a total surface area of 266 cm^2) were blended [Waring commercial blendor (Dynamic Corp, New Hartford, CT), speed set at level 5, for 1 min] with 75 mL of 0.1% peptone water. Decimal dilutions of the sample were made with 0.1% peptone water, and aliquots (0.1 mL) were plated in duplicate on a range of media. Potato Dextrose Agar (PDA, Difco, Detroit, MI), acidified with 10% tartaric acid to pH 3.5, and incubated at 25C for 5 days was used for yeast and mold enumeration. The FDA Bacteriological Analytical Manual, 7th Ed., (1992) method was used for enumeration of *E. coli*. Samples were plated on Violet Red Bile Agar (VRBA, Difco, Detroit, MI), with 5 mL overlay of the same agar containing 4-methylunbelliferyl-beta-D-glucuronide (MUG), and incubated at 37C for 24 h (Hitchins *et al.* 1992). Mesophilic aerobes were enumerated by plating on Plate Count Agar (PCA, Difco, Detroit, MI), incubated at 35C for 24 h (Messer *et al.* 1984). All plates were left at room temperature for 2 to 3 h prior to incubation at the appropriate temperature in order to allow injured organisms to repair (FDA BAM 1995). The choice of media and use of an incubation temerature of 25 ± 1C allowed for enumeration of other plant-associated organisms that may not grow well above this temperature. These organisms were estimated using Sabouraud Maltose Agar (SMA, Difco), incubated at 25C for 2 days.

Isolation and Characterization of Bacteria on SMA

After chlorine or hydrogen peroxide treatment and microbial deterimination, the SMA plates were flooded with 10 mL of sterile deionized H_2O to recover resistant microbes. The sample was vortexed, and a 1 mL sample was added to nutrient broth (Difco), incubated for 18 h, and then plated (0.1mL) on Pseudomonas isolation agar (Difco) for possible *Pseudomonas* spp (Andrews *et al.* 1992). Also possible *Salmonella* was checked using methods with slight modifications. The sample was diluted tenfold in selenite systine enrichment broth (Difco, Detroit, MI) and incuabted at 37C for 24 h, and 0.1 mL was surface-streaked on Salmonella-Shigella (SS). The SS plates were incubated at 37C and examined at 24 and 48 h for black or black-centered colonies. Also, biochemical tests including the oxidase test, Hugh Leifson, H_2S, urea agar, catalase, and Voges-Proskauer (VP) procedures were performed to presumptively identify the surviving organisms.

Data Analysis

Five replicate trials for each treatment were conducted. Data from each treatment were subjected to the Statistical Analysis System (SAS Institute, Cary, NC) for analysis of variance (ANOVA) and Bonferroni LSD method to determine significant differences within a given treatment over various sampling times.

RESULTS AND DISCUSSION

Natural Microflora

The surface microflora of cantaloupes were enumerated using various media incubated at two growth temperatures (Table 1). Total plate counts of surface microflora of the unwashed cantaloupes enumerated at 25C averaged 6.21 $\log_{10}$ cfu/cm^2 for mesophilic aerobes, 1.82 $\log_{10}$ CFU/cm^2 for coliforms, 0.51 $\log_{10}$ CFU/cm^2 for *E. coli*, and 3.30 $\log_{10}$ CFU/cm^2 for yeast and mold. Previous studies on aerobic mesophiles yielded similar microbial counts on unwashed cantaloupes (Ayhan *et al.* 1998; O'Connor-Shaw *et al.* 1994). The level of mesophilic aerobes

TABLE 1.
ESTIMATION OF THE SURFACE MICROFLORA OF CANTALOUPE[a]

	Incubation Temperature		
	25 C		35 C
Organisms	Log_{10} CFU/cm^2	Media	Log_{10} CFU/cm^2
Total aerobes	6.21 ± 0.23	PCA	6.89 ± 0.19
Total coliform	1.82 ± 0.17	VRBA	2.53 ± 0.13
E. coli	0.51 ± 0.11	VRBA + Mug	0.89 ± 0.05
Yeast/mold	3.30 ± 0.08	PDAA	1.16 ± 0.14
*Salmonella spp**	++	SMA	++
*Pseudomonas spp**	++	SMA	++

[a]Cell enumeration was after incubation at 25 C for 48 h, or at 35 C for 24 h.

Values represent means of 5 trials with duplicate determination ± standard deviation.

*These two organisms were presumed based on results of biochemical test. ++ = Presumptive positive test.

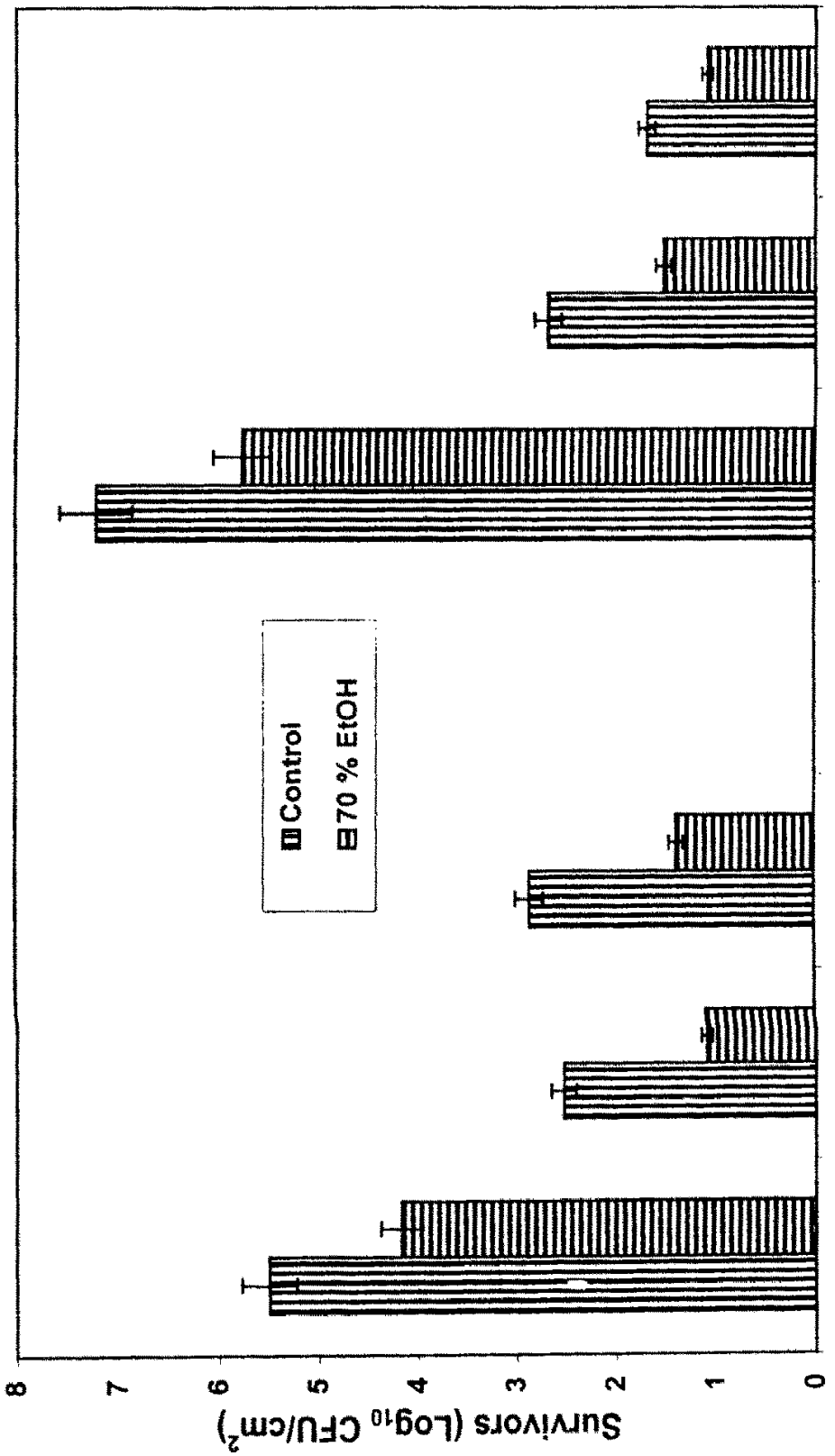

FIG. 1. RELATIONSHIP BETWEEN YEAST AND MOLD POPULATION ON SURVIVABILITY OF *E. COLI* ON CANTALOUPE SURFACE STORED AT 4C OR 20C FOR UP TO 120 H WITHOUT WASHING TREATMENTS
Values are means of five-determinations ± standard deviation.

and coliforms enumerated at 35C was slightly higher than the value at 25C. The organisms presumed to be *Salmonella* and *Pseudomonas* spp, isolated from the SMA media at the two incubation temperatures were present at trace levels. Of the 72 cantaloupes used for the study, only 4 canataloupes were presumptive for *Salmonella* while 13 cantaloupes were presumptive for *Pseudomonas spp.*

Effect of 70% EtOH on Natural Microflora

Treatment with 70% EtOH caused a 1.34 $\log_{10}$ CFU/cm^2 or 1.45 $\log_{10}$ CFU/cm^2 reduction of surface mesophilic aerobes enumerated on PCA media incubated at 25C or 35C, respectively (Fig. 1). Yeast and mold populations and the coliforms on the cantaloupes analyzed averaged 2.85 $\log_{10}$ CFU/cm^2 and 2.53 $\log_{10}$ CFU/cm^2 at 25C or 35C, respectively. Treatment with 70% EtOH, caused a 1.5 $\log_{10}$ CFU/cm^2 reduction at both temperatures for the total plate count and coliforms and a reduction of < 1.0 $\log_{10}$ CFU/cm^2 for yeast and mold populations at 35C. Most of the untreated and alcohol-treated cantaloupes had very low levels of *E. coli*(<0.5 $\log_{10}$ CFU/cm^2). Cantaloupes treated with 70% EtOH were adopted as controls and used for the inoculation studies to investigate *E. coli* attachment, survival and growth on the cantaloupe surface.

Inoculation Study

The results of the study designed to investigate influence of inoculum size on attachment of *E. coli* to the surface of the cantaloupes suggest that attachment is dependent on the inoculum size (Fig 2). With 10^4 to 10^6 CFU/mL *E.coli* inoculum, an average of 2.19 $\log_{10}$ CFU/cm^2 was deposited on the control or water washed cantaloupe surface. This number increased to 3.63 $\log_{10}$ CFU/cm^2 at 10^8 CFU/mL *E. coli* inoculum size. More bacterial attachment occured on cantaloupe surfaces sanitized with 70% EtOH. At 10^8-10^9 CFU/mL, bacterial attachment onto the cantaloupe surfaces averaged 4.4 $\log_{10}$ CFU/cm^2 and this value did not show any significant increase at higher inoculum size. Therefore, treatment of cantaloupes with 70% EtOH and 10^8 CFU/mL inoculum was adopted and used throughout the study.

Effect of Storage

There were significant changes in population of attached *E. coli* on the surface of the cantaloupe stored at room or refrigeration temperature over 120 h. Surviving populations of *E. coli* inoculated on cantaloupe surfaces decreased form 4.4 $\log_{10}$ CFU/cm^2 to approximately 3 $\log_{10}$ CFU/cm^2 on the surface of cantaloupe stored at 20C for 120 h (Fig. 3). No changes in *E. coli* population were observed on the surfaces of the cantaloupe stored at 4C for up to 120 h. The decline in *E. coli* population observed on the untreated cantaloupe surfaces (controls) stored at 20C for 120 h may be due to competitive outgrowth or antagonism by other microflora

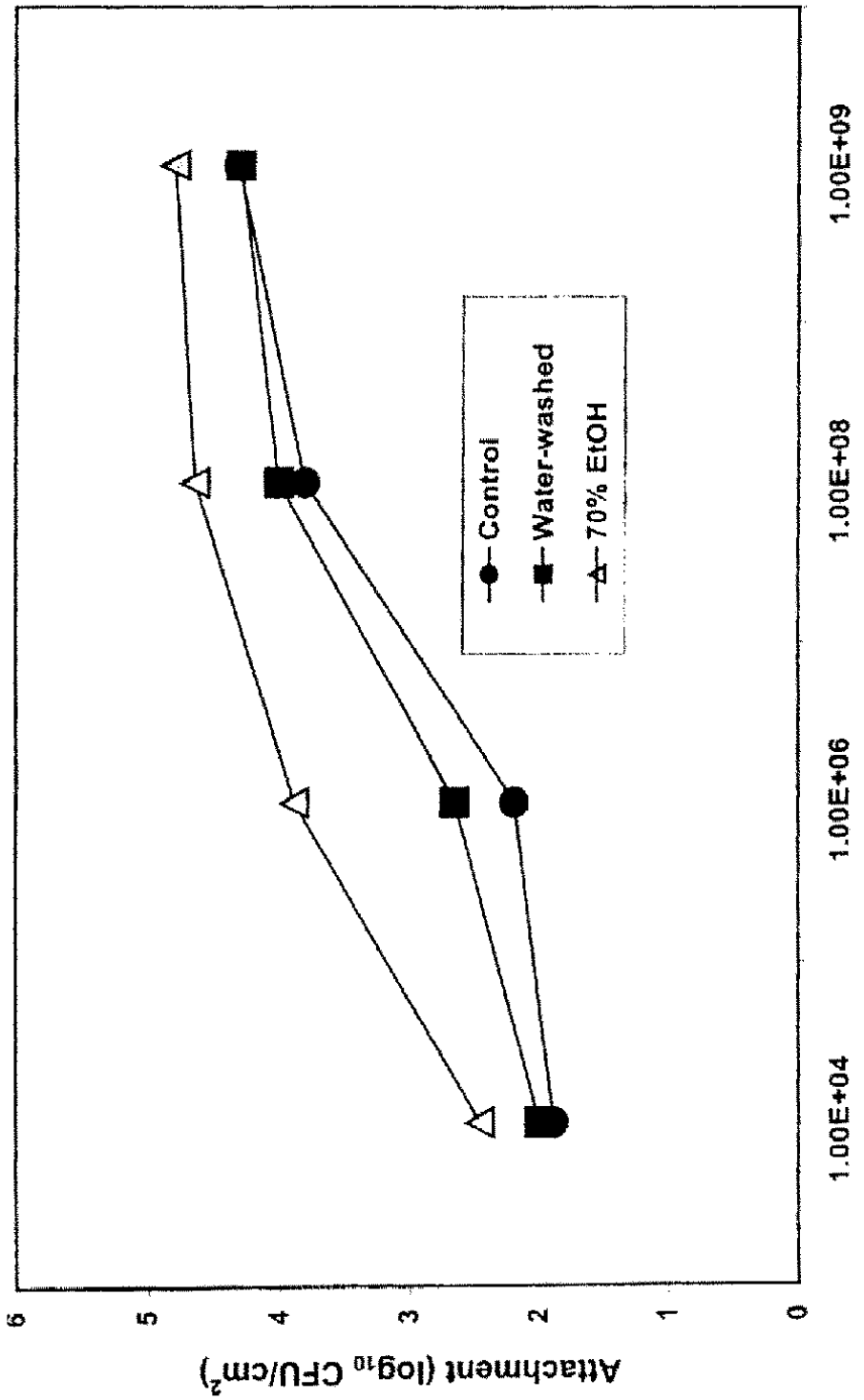

FIG. 2. STUDIES ON ATTACHMENT OF *E.COLI* ON CANTALOUPE SURFACES WASHED WITH WATER OR TREATED WITH 70% ETHANOL (EtOH)

Values are means of five-determinations ± standard deviation.

TABLE 2.
EFFECTS OF ALCOHOL ON REDUCTION OF AEROBIC PLATE COUNT (APC), YEAST AND MOLD POPULATION AND COLIFORMS ASSOCIATED WITH THE SURFACE OF CANTALOUPE[a]

	$\log_{10}$ CFU/cm^2		
Treatments	TPC	Yeast/mold	Coliforms
Control	5.69±0.24	3.52±0.17	2.81±0.05
70% EtOH	4.45±0.18	1.89±0.14	1.08±0.05

[a]Values represent means of five determinations with standard deviation.

(Janisiewicz *et al.* 1999) associated with cantaloupe surface. Other factors such as water stress, injuries or changes in surface properties of cantaloupe induced by the 70% EtOH treatment may have played an important role. For example the level of yeast and mold population on cantaloupe surfaces after 72 h storage at 20C increased from 2.4 to 3.5 $\log_{10}$ CFU/cm^2 (Fig. 4). This effect was not observed on surfaces of cantaloupe stored at 4C for up to 120 h suggesting inability of yeast to grow at this temperature. Also, the invasive ability of *E. coli* to establish themselves and grow on the cantaloupe surface may have been retarded due to the low storage temperature. Similar results on rind surfaces of melons stored at 5C have been reported (Del Rosario and Beuchat 1995). They also reported that growth of *E. coli* was observed on the rind of melons stored under high relative humidity at 25C for 14 days to 22 days.

Effect of Washing Treatments

The effect of storage and washing treatments on yeast and mold population of cantaloupes stored at 20C for up to 120 h is shown in Fig. 4. The increase in population could have resulted from moisture retained from the inoculation by submerging the whole cantaloupe in a bacterial inoculum. This phenomenon may have affected the survival of *E. coli* with the yeast and mold dominating the *E. coli* for space and available nutrient thus resulting to the decline of the later. Chlorine or hydrogen peroxide treatment were both effective in reducing the yeast and mold population throughout storage. However, hydrogen peroxide appeared superior to chlorine.

The effect of storage and washing treatments on the *E. coli* population of inoculated cantaloupes stored at 20C for up to 120 h is shown in Fig. 5. The population of *E. coli* on the cantaloupe surfaces gradually declined as storage time increased. All cantaloupe surfaces appeared dry and flaky after 24 h storage, and the *E. coli* appeared to be easliy detached. The *E. coli* decline was unexpected.

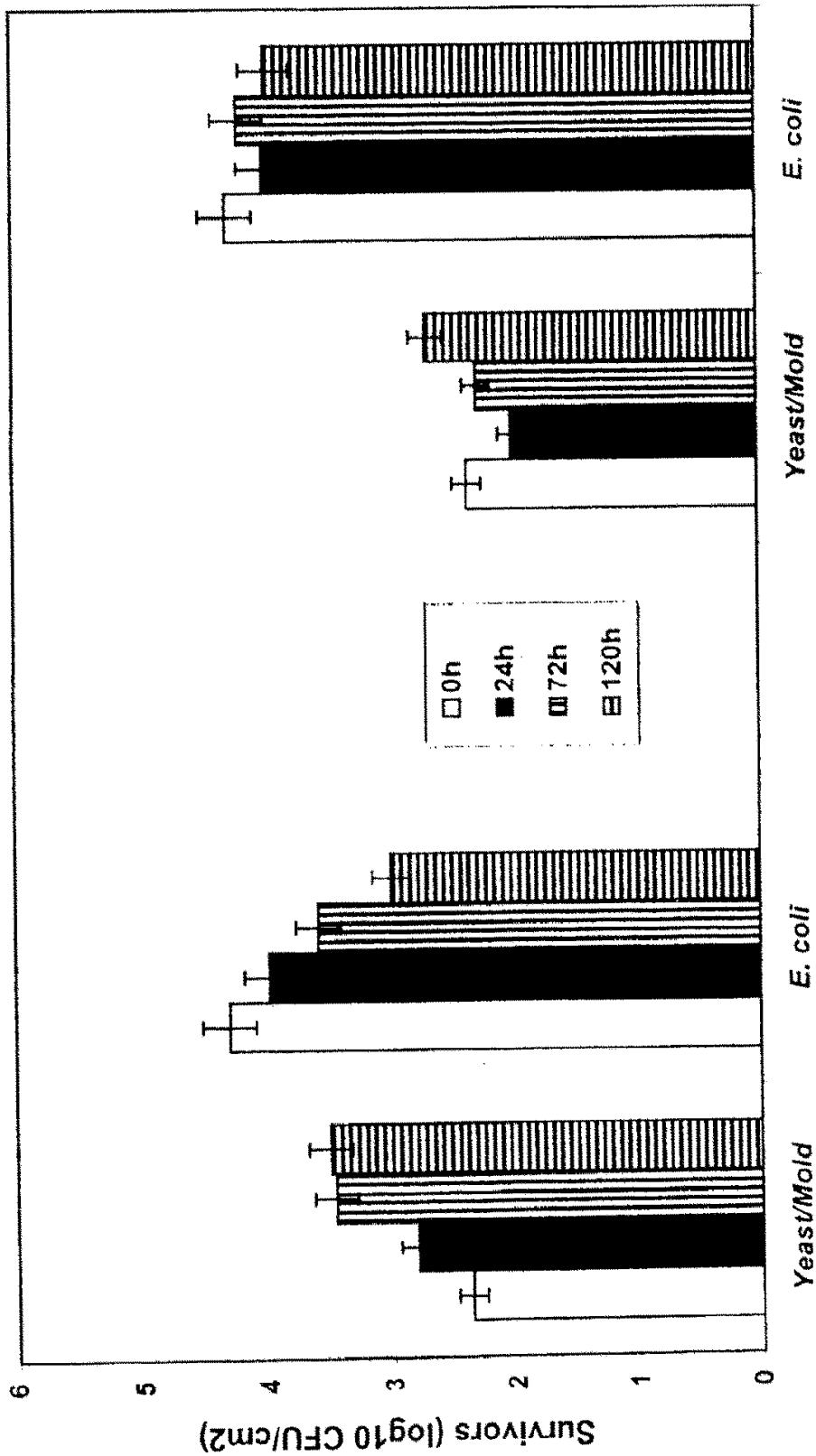

FIG. 3. EFFECT OF STORAGE TEMPERATURES ON SURVIVAL OF MESOPHILIC AEROBES, YEAST AND MOLD POPULATION AND THE INOCULATED *E. COLI* POPULATION ATTACHED ON THE SURFACE OF CANTALOUPE STORED AT 4C OR 20C FOR 120 H
Values are means of five-determinations ± standard deviation.

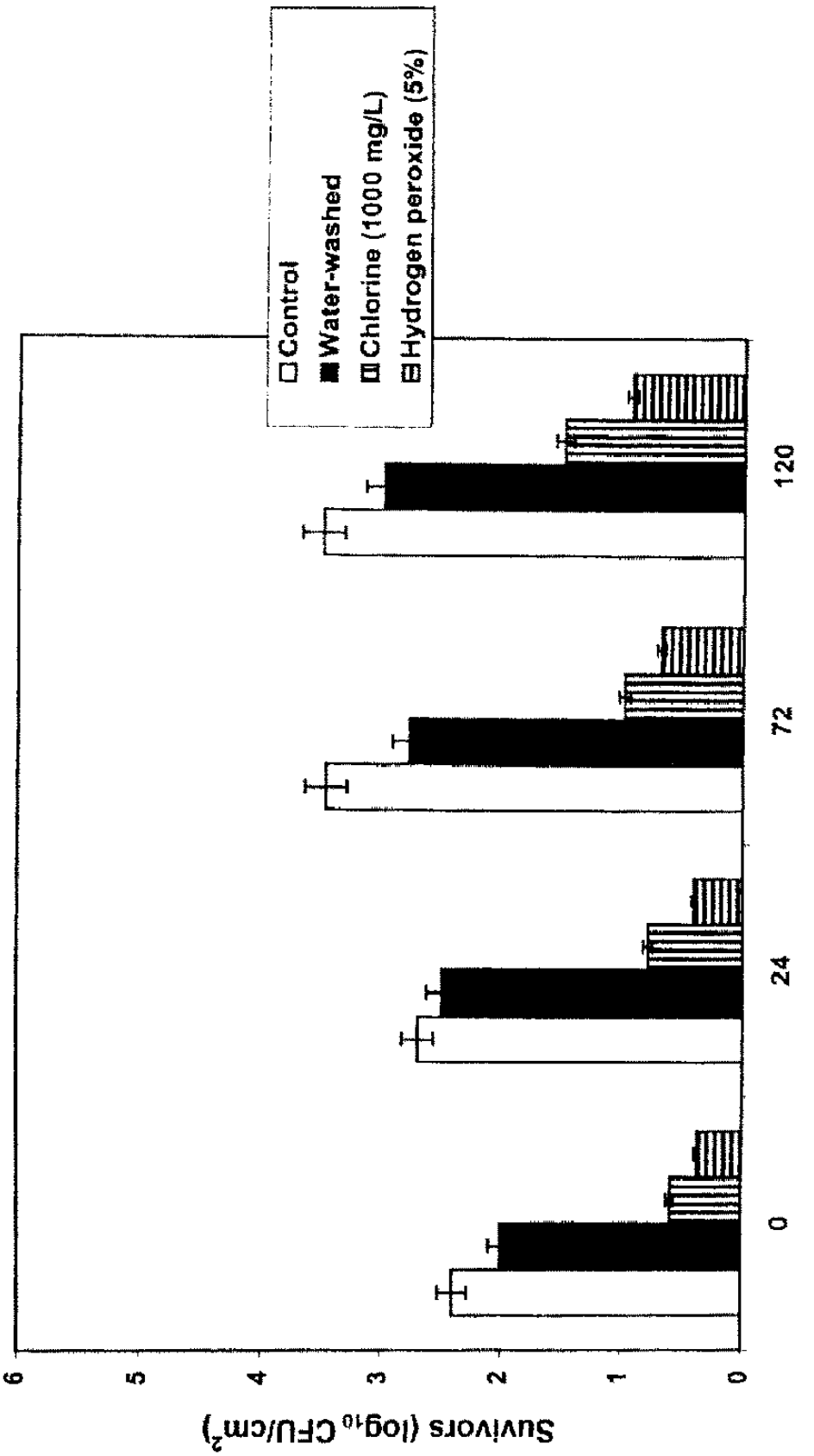

FIG. 4. EFFICACY OF WASHING TREATMENTS ON THE YEAST AND MOLD POPULATION OF SURFACE CANTALOUPE STORED AT 20C FOR UP TO 120 H

Values are means of five-determinations ± standard deviation.

After 24 h storage at 4C, chlorine and hydrogen peroxide treatment resulted in a 3.2 $\log_{10}$ CFU/cm^2 and a 1.7 $\log_{10}$ CFU/cm^2 reduction of *E. coli* on the cantaloupe surface, respectively. After 24 h storage at 4C, results of washing treatments appeared different from the 0 h to 24 h data. The *E. coli* on the surfaces of cantaloupe appeared more resistant to the washing treatments. At longer storage (20C) intervals proir to treatment (Fig. 6), population reductions declined, and at 120 h, both sanitizers were ineffective. This is probably due to biofilm formation or other physical changes (dehydration) that may have occurred due to alcohol treatment. Despite the application of the washing treatments, the number of survivors remains approximately consistent suggesting stronger attachment to the melon surfaces or possibly biofilm formation.

Although chlorine or hydrogen peroxide appeared to partially decontaminate *E. coli* attached on the cantaloupe surface, total decontamination was not achieved. Other studies (Beuchat 1995; Brakett 1992) on chlorine treatment of vegetables reported incomplete removal or inactivation of bacteria on fresh produce. This condition may be more representative of commercial practice if contamination with *E. coli* occurred prior to or during harvest. The efficacy of washing treatments on detachment or inactivation of *E. coli* on cantaloupe surfaces is dependent on the state and location of the orgaisms on the outer surface of the cantaloupes. Seo and Frank (1999) reported protection of microorganisms embedded in tissue from chemicals such as chlorine that have little penetrating power.

Irregularities such as roughness, crevices, and pits have been shown to increase bacterial adherence by increasing cell attachment and reducing the ability to remove cells (Austin and Bergeron 1995; Frank and Koffi 1990; ICMS 1980). This could account for the variability in total plate counts of surface microflora associated with cantaloupes and the large number of survivors after treatment with chlorine and hydrogen peroxide. At the supermarket, where the consumer handles the cantaloupes in the process of buying, the likelihood of increasing the population of surface microflora of cantaloupe through transfer from human skin and from cantaloupe to cantaloupe is increased. Both chlorine and hydrogen peroxide were effective in reducing the surface microflora of cantaloupe, as enumerated with all the media used. Organisms that survived the chlorine or the hydrogen peroxide treatment and recovered in SMA were isolated and characterized. Most of these organisms were presumptive *Pseudomonas* based on their color reaction (first the colonies were pink, then changed to maroon dark red, and finally turned black) during oxidase test.

Chlorine and hydrogen peroxide are powerful oxidizing compounds which rapidly react with proteins of microorganisms, hence leading to interference in enzyme metabolism of the microbial cell (Baker 1926; Trueman 1971). *Salmonella, Proteus* and *Pseudomonas* strains were reported to be more resistant to chlorine than other organisms (Madden 1992; Yildiz 1994). Our data seem to confirm this, since the majority of the organisms isolated from the chlorine or hydrogen peroxide treated samples plated on SMA media were presumptive

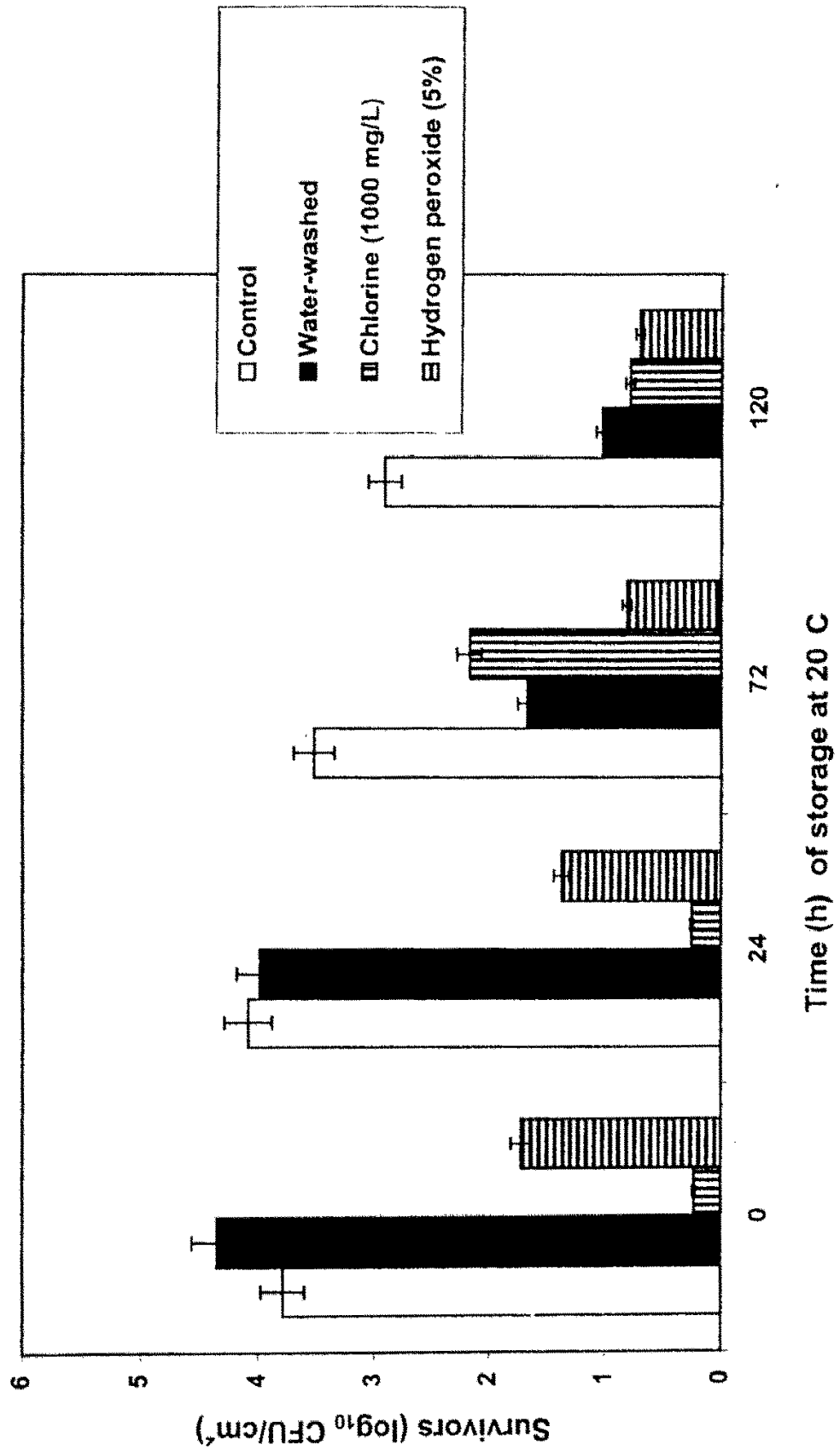

FIG. 5. EFFECT OF WASHING TREATMENT ON SURVIVAL OF INOCULATED *E. COLI* POPULATION ATTACHED ON THE SURFACE OF CANTALOUPE STORED AT 4C FOR UP TO 120 H
Values are means of five-determinations ± standard deviation.

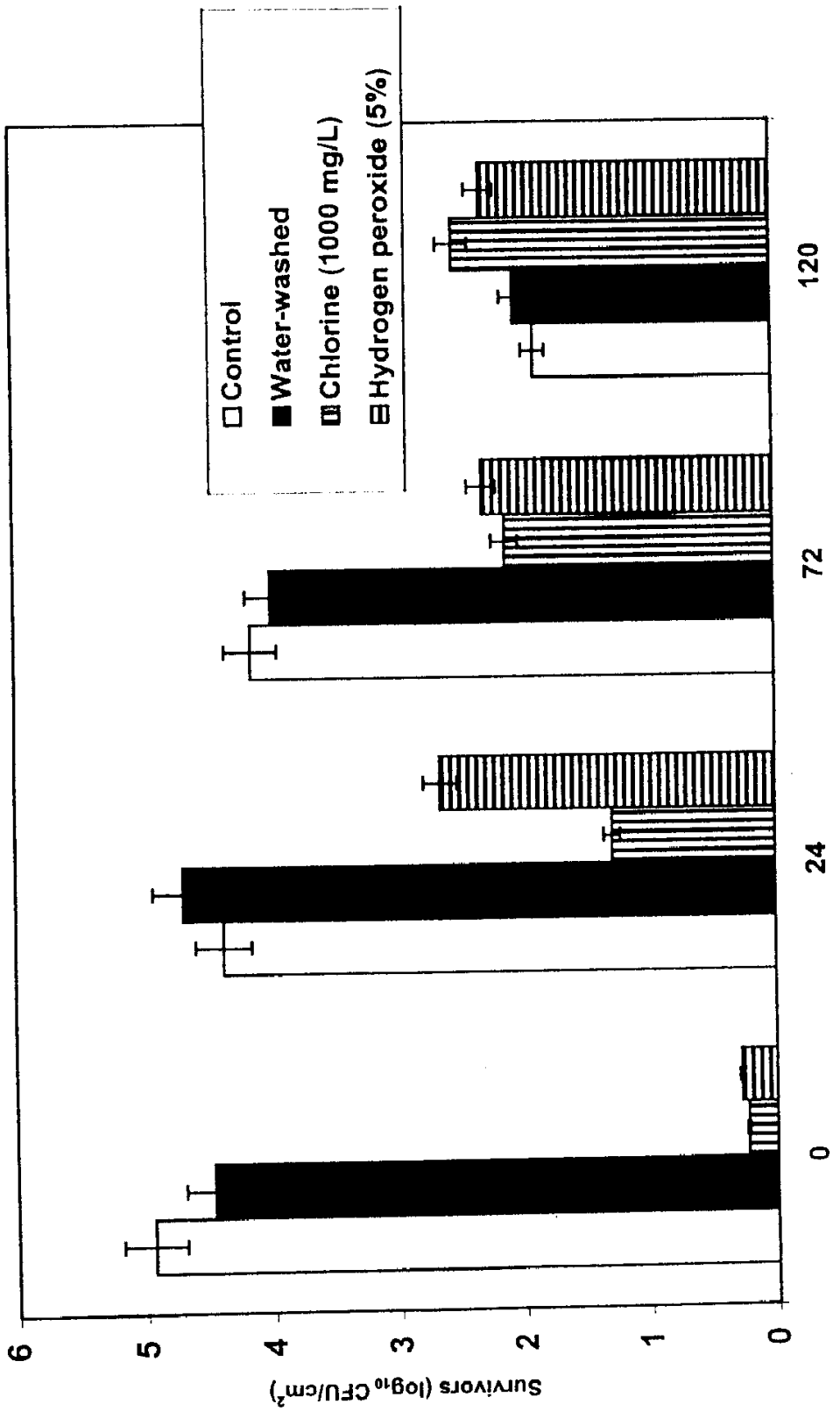

FIG. 6. EFFECT OF WASHING TREATMENT ON SURVIVAL OF INOCLUATED *E. COLI* POPULATION ATTACHED ON THE SURFACE OF CANTALOUPE STORED AT 20C FOR UP TO 120 H
Values are means of five-determinations ± standard deviation.

Pseudomonas or *Salmonella* spp. FDA field surveys of imported cantaloupe and watermelon have shown that there is a low incidence of *Salmonella* on the exterior surface of intact melon (Murphy 1999).

CONCLUSION

Surface treatment with 70% EtOH resulted in approximately 1.5 $\log_{10}$ CFU/cm^2 reduction of surface mesophilic aerobes of cantaloupes, suggesting that this treatment is an inadequate means of surface sterilization. The efficacy of chlorine and hydrogen peroxide in reducing the population of *E. coli* on inoculated cantaloupe was dependent on the interval between inoculation and application of the washing treatment. Chlorine treatment appeared to be more effective than hydrogen peroxide in reducing the *E. coli* populations attached on the cantaloupe surfaces within 24 h of inoculation. If bacterial attachment occurred more than 24 h prior to washing, detachment or inactivation using chlorine or hydrogen peroxide treatments was less effective, and the difference between the two treatments diminished.

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